

### Remarks

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

This submission is accompanied by a Request for Continued Examination, a petition for extension of time, and an information disclosure statement. All fees should be withdrawn from Deposit Account 14-1138.

Claim 1 has been amended to recite higher stringency requirements (i.e., structural requirements of the claimed DNA molecule based on hybridization capability) as well the functional requirements of the encoded single-strand binding protein subunit ("binds to single-stranded DNA"). A form of the latter limitation also appears in original claim 9. Claims 10 and 11 have been cancelled.

Claims 1, 2, 6-9, and 12-21 are pending, and claims 17-21 stand allowed.

The objection to the specification is respectfully traversed in view of the above claim amendments and the following remarks. The specification of parent application 09/057,416 and the present application both recite a number of stringency conditions that can be used (at page 30, lines 7-21), and clearly indicate that stringency of the hybridization conditions can be further modified by altering, among other variables, the salt or temperature. Specific support for the temperature and salt conditions presently recited in claim 1 are also provided at page 35, lines 19-29 (of both the present application and parent application 09/716,964), which also indicates that higher stringency washes can be utilized. Therefore, both the present application and the parent application provide descriptive support for the claim language. Because this language is not new matter, the objection to the specification should be withdrawn.

The rejection of claims 1, 2, and 6-16 under 35 U.S.C. § 112 (first paragraph) as lacking written descriptive support is respectfully traversed.

The PTO has asserted at page 4 of the outstanding office action that the "function" of the encoded single-strand binding protein remains in question. Applicants respectfully disagree.

Claim 1 presently recites that "the encoded single-strand binding protein binds to single-stranded DNA." Persons of skill in the art would appreciate that this is precisely the

function attributed to single-strand binding proteins that cooperate with polymerase in general, including polymerase III enzyme complexes. Indeed, the single-strand binding protein of *E. coli* was previously shown to bind cooperatively to single-stranded DNA and destabilize helical duplexes, causing a lowering of the melting temperature (*see* Kunkel et al., "Single-strand Binding Protein Enhances Fidelity of FNA Synthesis *in vitro*," *Proc. Natl. Acad. Sci. USA* 76(12):6331-6335 (1979) ("Kunkel") at p. 6331 (copy attached hereto as **Exhibit 1**)). Kunkel even reports that the *E. coli* single-strand binding protein, when used with the *E. coli* Pol III enzyme complex and DNA polymerases of *divergent sources*, was capable of increasing fidelity by as much as 10-fold. Kunkel at abstract.

Use of single-strand binding proteins of the present invention in combination with a Pol III-type enzyme is clearly contemplated (*see* page 44, lines 17-19, and page 45, lines 2-4 ("The reaction is incubated at elevated temperature ... and could include other proteins to enhance activity such as a single strand DNA binding protein.")). This combination would be expected to function in a similar manner to other prior art single-strand binding proteins. Use of the DNA molecules to produce the encoded single-strand binding proteins is also contemplated.

Given the shared function among single-stranded binding proteins, persons of skill in the art would have expected a shared structure/function relationship to exist for these proteins and their encoding nucleic acids. In fact, De Vries et al. reported as earlier as 1994 that the N-terminal amino acid sequences of single-strand binding proteins are highly conserved within the first two-thirds of the protein sequence and highly divergent within the C-terminal third. De Vries et al., "The Single-stranded-DNA-binding Proteins (SSB) of *Proteus mirabilis* and *Serratia marcescens*," *Eur. J. Biochem.* 224:613-622 (1994) at abstract and Fig. 2 (copy attached as **Exhibit 2**)). Thus, persons of skill in the art would have expected both structural and functional conservation among single-strand binding proteins and their encoding nucleic acids.

That the encoded single-strand binding protein is structurally related to other single-strand binding proteins is evident from the comparison of the *Thermus thermophilus* single-strand binding protein of SEQ ID NO: 172 to the *B. stearothermophilus* single-strand binding protein of SEQ ID NO: 176 (*see* page 61, line 32, to page 62, line 2). These two species show about 23 percent identity over their length.

From the foregoing, persons of skill in the art would have expected members of the claimed genus of DNA molecules encoding single-stranded binding proteins to possess both similar function and structure. Indeed, even higher similarity among the encoded single-strand

binding proteins would be expected among more closely related bacteria, and that is precisely what applicants demonstrated in the previous response (*see* Exhibits 1-3 attached to the November 27, 2006, amendment). Thus, given applicants prior demonstration of structural similarity among homologous single-strand binding protein subunits of *Bacillus* (and *Geobacillus*), applicants respectfully submit that the genus of isolated DNA being claimed is adequately represented by the species of SEQ ID NO: 175.

With regard to claim 9, applicants submit that the same expectation would apply to nucleic acid molecules isolated from *Bacillus* (now *Geobacillus*) *stearothermophilus* variants. That is, given the relatedness of the organisms, persons of skill in the art would expect that the nucleic acids encoding SSB proteins would be structurally similar and encode functional equivalents. Thus, the species of SEQ ID NO: 175 would be expected to represent the small genus of variants encompassed by the claim.

In view of all of the foregoing, applicants submit that the rejection of claims 1, 2, and 6-16 is improper and should be withdrawn.

The rejection of claims 1, 2, and 6-16 under 35 U.S.C. §112 (first paragraph) for lack of enablement is respectfully traversed.

It is the position of the PTO that the specification does not provide sufficient guidance for making and using other DNA molecules encoding single-strand binding proteins within the scope of the claims. Applicants respectfully disagree.

The present application provides the nucleotide sequence of *Bacillus stearothermophilus ssb* (e.g., SEQ ID NO: 175) and describes how one of ordinary skill can isolate homologs of the disclosed sequence (*see* page 41, line 9 to page 42, line 29; Example 12), express the SSB protein encoded by such homologous *ssb* sequences (*see* Example 23, purifying *Aquifex* SSB protein), and test the encoded SSB protein for activity (*see* Examples 26 and 30, using *Aquifex* SSB protein in assay). Thus, one of ordinary skill in the art would have been fully able to make and use DNA molecules (and their encoded proteins) within the scope of the presently claimed invention.

Moreover, with regard to method 3 for homolog identification, described at page 42, that is precisely the approach used to identify the *ssb* homologs shown in Exhibit 1 of applicants' November 27, 2006, submission (i.e., from other *Bacillus* or *Geobacillus* organisms). For this reason, it should be apparent that the present application fully enables the production and

use of other species of *Bacillus* or *Bacillus* (now *Geobacillus*) *stearothermophilus* SSB-encoding DNA molecules.

For these reasons, applicants submit that the rejection of claims 1, 2, and 6-16 for lack of enablement is improper and should be withdrawn.

In view of all of the foregoing, applicant submits that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

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**Exhibit 1: Kunkel et al., *Proc. Natl. Acad. Sci. USA* 76(12):6331-6335 (1979)**

## Single-strand binding protein enhances fidelity of DNA synthesis *in vitro*

(accuracy/DNA polymerase/DNA binding protein/base selection)

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**ABSTRACT** The effect of *Escherichia coli* single-strand binding protein on the accuracy of *in vitro* DNA synthesis has been determined by using two independent methods. By using the synthetic polynucleotide poly(d(A-T)) and measuring dGTP misincorporation or by using  $\phi$ X174 DNA and measuring nucleotide substitutions, we found that binding protein increases the fidelity of DNA synthesis by as much as 10-fold. This increase is observed with DNA polymerases of divergent sources and is progressive with increasing concentration of binding protein. The increased accuracy observed with DNA polymerases lacking a 3'  $\rightarrow$  5' exonuclease points to a mechanism other than augmented proofreading. In accord with the properties of single-strand binding proteins, it is suggested that increased fidelity is a result of enhanced base selection by the DNA polymerase, resulting from increased rigidity of the template due to its interaction with binding protein.

On the basis of spontaneous mutation rates, the accuracy of DNA replication *in vivo* is estimated to be  $10^{-1}$  to  $10^{-11}$  stable misincorporations per base pair (1). This accuracy is several orders of magnitude greater than that measured with purified DNA polymerases *in vitro*, typically  $10^{-3}$  to  $10^{-6}$  (2, 3). The cellular mechanisms used to achieve this enhanced fidelity are unknown, although several mechanisms have been suggested (3, 4). In our continuing effort to understand the cellular mechanisms for the accuracy of DNA replication, we have begun a systematic study of the influence of proteins known to be required for DNA replication on the fidelity of DNA synthesis *in vitro*. Of particular concern was single-strand binding protein (SSB), first purified by Alberts and Frey from *T<sub>4</sub>*-infected *Escherichia coli* (5) and later from uninfected *E. coli* (6). SSB has been shown to be essential for DNA replication (5, 7-9) and to be involved in the processes of recombination and repair (ref. 10, unpublished results). SSB binds cooperatively to single-stranded DNA and destabilizes helical duplexes, causing a lowering of the melting temperature (5). These characteristics have been used to isolate similar DNA binding proteins from several eukaryotic systems (11-15), although their role in DNA metabolism has not been firmly established.

A role for SSB in modulating the accuracy of DNA replication has been suggested by several *in vivo* studies demonstrating that mutations in the gene for binding protein alter the overall mutation frequency of bacteriophage T<sub>4</sub> (16-18). In addition, during the process of copying poly(d(A-T)) with *T<sub>4</sub>* DNA polymerase, Cillín and Nossal (19) showed a 30-40% reduction in turnover of noncomplementary nucleotides *in vitro* upon the addition of *T<sub>4</sub>* gene 32 protein (SSB). Also, Liu *et al.* (9) estimated that the error rate of copying  $\phi$ X174 DNA by using the *T<sub>4</sub>* replication complex (seven proteins), which includes SSB, approaches the mutation rate *in vivo*. We therefore desired to

assess directly the contribution of SSB to the accuracy of DNA synthesis. We report here our findings that purified *E. coli* SSB increases the accuracy of *in vitro* DNA synthesis by more than an order of magnitude, measured in two independent *in vitro* assay systems. Thus, a protein—other than DNA polymerase and essential to DNA replication—functions in enhancing base selection.

### MATERIALS AND METHODS

**Materials.** Unlabeled nucleotide triphosphates were purchased from P.L. Biochemicals, New England Nuclear was the source of [ $\alpha$ -<sup>32</sup>P]dATP, [ $\alpha$ -<sup>32</sup>P]dTTP, and [<sup>3</sup>H]dGTP (11,000 cpm/pmol). Poly(d(A-T)), containing only  $1 \pm 0.5$  mol of dGMP for every  $2 \times 10^6$  mol of dAMP and dTMP, was synthesized as described (20). *E. coli* DNA polymerase (Pol III) was purified by a modification of the procedure of McHenry and Crow (21). Electrophoresis in the presence of sodium dodecyl sulfate confirmed the presence of the  $\alpha$ ,  $\epsilon$ , and  $\beta$  subunits. *E. coli* DNA Pol I (22), avian myeloblastosis virus (AMV) DNA polymerase (23), DNA polymerase- $\alpha$  from acute lymphoblastic leukemia cells (24), and Novikoff hepatitis DNA polymerase- $\beta$  (25) were purified as described. Calf thymus DNA polymerase- $\alpha$  was a generous gift of Bethesda Research Laboratories (Rockville, MD), and *T<sub>4</sub>* DNA polymerase was supplied by P. England (Johns Hopkins University, Baltimore, MD) and B. M. Alberts (University of California, San Francisco, CA). SSB was purified to >96% homogeneity from *E. coli* strain HMS 83 by the method of Weiner *et al.* (8) or by a simple procedure utilizing blue dextran-sepharose chromatography followed by heating to 100°C (unpublished results). The elimination of any DNA affinity column yields essentially DNA-free preparations of SSB.

**Fidelity Assays.** Fidelity assays with poly(d(A-T)) as a template were performed as described in the legends to Tables 1-4. In these assays, the error rate is defined as the ratio of incorrect to total correct deoxynucleotides incorporated. The DNA polymerase reactions for copying  $\phi$ X174 DNA are as described in the legend to Table 5. A detailed account of the entire methodology for the  $\phi$ X174 fidelity assay has been published (26), as was the method used to calculate the error rate from the observed reversion frequency of copied versus uncopied DNA (27).

### RESULTS

**Fidelity of DNA Pol III.** As a first step in assessing the contribution of *E. coli* SSB to fidelity, we measured the accuracy of copying poly(d(A-T)) by purified *E. coli* DNA Pol III. This enzyme was chosen because *E. coli* SSB is required for DNA

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Abbreviations: SSB, single-strand binding protein; Pol I and Pol III, DNA polymerase I and III, respectively; AMV, avian myeloblastosis virus.

replication and Pol III has been shown to be the replicative DNA polymerase in *E. coli* (28). Pol III copies poly(dA-T) with an error rate of about 1/30,000, using 5 mM Mn<sup>2+</sup> as a metal activator (Table 1). This accuracy is greater than that reported for Pol III\* in copying poly(dA)-oligo(dT)<sub>12-15</sub> in Mg<sup>2+</sup>-activated reactions (30) and less than that reported for Pol III in copying poly(dA)-oligo(dT)<sub>12-15</sub> with 1 mM Mn<sup>2+</sup> (31). Synthesis with Mg<sup>2+</sup> on poly(dA-T) was insufficient for quantitation of fidelity. The optimal Mn<sup>2+</sup> concentration for Pol III (5 mM) was much greater than that reported with phage DNA polymerase (20, 23, 29, 32). Moreover, the error rate was not increased at a Mn<sup>2+</sup> concentration as great as 20 mM (results not given). Omission of Mn<sup>2+</sup>, dTTP, or, most importantly, poly(dA-T) eliminated incorporation of both correct and incorrect nucleotides. Thus, the enzyme preparation was devoid of contaminating DNA, which could act as a template for incorporation of sufficient amounts of dGMP to interfere with any measurements of fidelity. Analysis of the product of the reaction synthesized with DNA polymerase from *E. coli* (Pol I) (30), AMV (23), and human placenta ( $\alpha$  and  $\beta$ ) (33) indicates that this assay measures internal misincorporation of dGMP present predominantly as single-base substitutions.

Due to the high accuracy of Pol III even with Mn<sup>2+</sup>, any further increase in accuracy would be difficult to quantify. We therefore defined a set of error-prone conditions for the enzyme by simply increasing the concentration of incorrect nucleotide relative to correct nucleotide substrates in the reaction. As shown in Table 2, the increase in error rate of Pol III is directly proportional to the increase in the concentration of the incorrect nucleotide. These results substantiate previous findings on the effects of biased substrate pool sizes with Pol I (20, 26), AMV DNA polymerase (23, 29), and T<sub>4</sub> DNA polymerase (19, 32) and are in accord with kinetic models on the fidelity of DNA synthesis (34).

Effect of SSB on Fidelity of Pol III. The results of addition of increasing amounts of SSB to Pol III-catalyzed poly(dA-T) assays are shown in Table 3. In experiment 1, equal concentrations of incorrect and correct substrates were used. In ex-

Table 2. Proportionality of error rate of *E. coli* DNA Pol III to concentration of incorrect nucleotide

| dGTP concentration, $\mu$ M | Nucleotide incorporated, pmol |           | Error rate | Relative increase in error rate |
|-----------------------------|-------------------------------|-----------|------------|---------------------------------|
|                             | Correct                       | Incorrect |            |                                 |
| 50                          | 973                           | 0.028     | 1/33,800   | 1.0                             |
| 100                         | 880                           | 0.056     | 1/15,800   | 2.2                             |
| 175                         | 638                           | 0.065     | 1/9,700    | 3.4                             |
| 250                         | 444                           | 0.068     | 1/6,530    | 5.1                             |

Assays were performed as described in the legend to Table 1 with 5 mM MnCl<sub>2</sub> and increasing concentrations of [PH<sub>3</sub>]dGTP. As indicated, to a maximum of 250  $\mu$ M. In each experiment, concentration of dATP and dTTP was 50  $\mu$ M. All assays were carried out in triplicate. Incorporation in the absence of incubation was determined with each concentration of [PH<sub>3</sub>]dGTP and the values obtained were subtracted. Incorporation of dGTP in the absence of incubation varied from 0.003 to 0.011 pmol at 50–250  $\mu$ M [PH<sub>3</sub>]dGTP.

periment 2, the concentration of the incorrect nucleotide was 5-fold greater than that of either correct nucleotide. In both instances, the addition of binding protein in amounts stoichiometric with DNA resulted in a 15–27% increase in the rate of polymerization, whereas greater amounts were inhibitory, as indicated by previous reports (6, 35). Most importantly, SSB increased the accuracy of Pol III-catalyzed DNA synthesis in a concentration-dependent manner. The greatest effect shown here, a 5.8-fold increase in fidelity, was observed at an inhibiting concentration of SSB. This amount of SSB (weight ratio SSB to DNA of 5:1) is slightly less than that required to coat all nucleotides if the template were completely single-stranded. Inhibition of synthesis by greater amounts of SSB prevented quantitation of fidelity. By linear extrapolation of the results obtained with biased pools to the results obtained with equal nucleotide concentrations of incorrect and correct nucleotides, we find that the error rate of Pol III with SSB is less than 1/200,000.

Effect of SSB on Fidelity of Other DNA Polymerases. In order to determine whether the increase in accuracy was limited to a specific reaction of Pol III with *E. coli* SSB, we examined the effect of SSB on the fidelity of DNA synthesis catalyzed by other DNA polymerases (Table 4). The fidelity was increased severalfold with all DNA polymerases studied with either Mg<sup>2+</sup> or Mn<sup>2+</sup> as a metal activator. The increase in fidelity observed

Table 1. Fidelity of *E. coli* DNA Pol III in copying poly(dA-T)

| Condition                 | Nucleotide incorporated, pmol |           | Error rate |
|---------------------------|-------------------------------|-----------|------------|
|                           | Correct                       | Incorrect |            |
| I. Complete               |                               |           |            |
| 1 mM MnCl <sub>2</sub>    | 402                           | 0.001     | 1/40,200   |
| 2 mM MnCl <sub>2</sub>    | 524                           | 0.020     | 1/31,200   |
| 3 mM MnCl <sub>2</sub>    | 808                           | 0.021     | 1/38,500   |
| II. Complete              |                               |           |            |
| (5 mM MnCl <sub>2</sub> ) | 1785                          | 0.069     | 1/30,300   |
| – MnCl <sub>2</sub>       | <1.0                          | <0.001    | —          |
| – poly(dA-T)              | <1.0                          | 0.006     | —          |
| – dTTP                    | 2.3                           | 0.008     | —          |

Assays were performed in a 50- $\mu$ l volume containing 20 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 1  $\mu$ g of poly(dA-T), 50  $\mu$ M each of dATP, [ $\alpha$ -<sup>32</sup>P]dGTP (5–20 dpm/pmol) (1 dpm = 16.7 mBq), and [PH<sub>3</sub>]dGTP (11,500 cpm/pmol), 100  $\mu$ g of bovine serum albumin per ml, 100  $\mu$ M ATP, the indicated concentration of MnCl<sub>2</sub>, and 0.2 (Exp. I) or 0.4 (Exp. II) units of *E. coli* DNA Pol III. For the experiment in which dTTP was omitted, [ $\alpha$ -<sup>32</sup>P]dGTP (5.8 dpm/pmol) was used as the labeled correct nucleotide. Incubation was for either 30 or 60 min at 37°C. Acid-insoluble radioactivity was determined after repeatedly precipitating the polynucleotide product with 1 M hydrochloric acid/0.05 M sodium pyrophosphate and redissolving with 0.2 M NaOH (29). All assays were performed in triplicate and the average incorporation was obtained after subtracting the amount of incorporation in the absence of incubation (typically 15–20 dpm for <sup>32</sup>P and 50–100 cpm for <sup>3</sup>H).

Table 3. Effect of *E. coli* SSB on fidelity of *E. coli* DNA Pol III

| SSB/DNA*                  | Nucleotide incorporated, pmol |           | Error rate | Relative decrease in error rate |
|---------------------------|-------------------------------|-----------|------------|---------------------------------|
|                           | Correct                       | Incorrect |            |                                 |
| Exp. 1 (50 $\mu$ M dGTP)  |                               |           |            |                                 |
| —                         | 1034                          | 0.032     | 1/32,000   | 1.0                             |
| 0.6:1                     | 1196                          | 0.030     | 1/39,800   | 1.9                             |
| 1.2:1                     | 1314                          | 0.017     | 1/77,300   | 2.4                             |
| 2.6:1                     | 796                           | 0.006     | 1/132,500  | 4.1                             |
| Exp. 2 (250 $\mu$ M dGTP) |                               |           |            |                                 |
| —                         | 657                           | 0.086     | 1/7,580    | 1.0                             |
| 0.5:1                     | 750                           | 0.060     | 1/12,500   | 1.6                             |
| 1.2:1                     | 763                           | 0.060     | 1/15,300   | 2.0                             |
| 2.5:1                     | 638                           | 0.028     | 1/22,800   | 3.0                             |
| 5:1                       | 399                           | 0.009     | 1/44,200   | 5.8                             |

Assays were performed as described in the legends to Table 1 and 2 with 5 mM MnCl<sub>2</sub> and the indicated amounts of *E. coli* SSB.

\* The ratio of SSB to DNA is a weight ratio. Saturation is calculated at 6:1, assuming one SSB molecule (74,000 molecular weight) covers 35 nucleotides (6).

Table 4. Effect of *E. coli* SSB on fidelity of different DNA polymerases

| SSB/DNA                                    | Metal activator   | Nucleotide incorporated, pmol |           | Error rate | Relative decrease in error rate |
|--|-------------------|-------------------------------|-----------|------------|---------------------------------|
|  |                   | Correct                       | Incorrect |            |                                 |
| <i>E. coli</i> DNA Pol I                   |                   |                               |           |            |                                 |
| —  | MgCl <sub>2</sub> | 274                           | 0.011     | 1/24,900   | 1.0                             |
| 0.5:1                                      | MgCl <sub>2</sub> | 327                           | 0.009     | 1/68,600   | 2.4                             |
| 1.25:1                                     | MgCl <sub>2</sub> | 698                           | 0.010     | 1/68,600   | 2.8                             |
| —  | MnCl <sub>2</sub> | 227                           | 0.288     | 1/769      | 1.0                             |
| 1.25:1                                     | MnCl <sub>2</sub> | 410                           | 0.108     | 1/3,900    | 4.0                             |
| <i>T<sub>4</sub></i> DNA polymerase        |                   |                               |           |            |                                 |
| —  | MgCl <sub>2</sub> | 169                           | 0.004     | 1/42,300   | 1.0                             |
| 0.5:1                                      | MgCl <sub>2</sub> | 256                           | <0.001    | <1/256,000 | >8.1                            |
| 1.25:1                                     | MgCl <sub>2</sub> | 334                           | 0.001     | 1/334,000  | 7.9                             |
| —  | MnCl <sub>2</sub> | 195                           | 0.003     | 1/65,000   | 1.0                             |
| 1.25:1                                     | MnCl <sub>2</sub> | 885                           | 0.001     | 1/885,000  | 13.6                            |
| AMV DNA polymerase                         |                   |                               |           |            |                                 |
| —  | MgCl <sub>2</sub> | 189                           | 0.078     | 1/2,420    | 1.0                             |
| 0.5:1                                      | MgCl <sub>2</sub> | 691                           | 0.076     | 1/8,910    | 3.6                             |
| 1.25:1                                     | MgCl <sub>2</sub> | 628                           | 0.064     | 1/11,800   | 4.8                             |
| —  | MnCl <sub>2</sub> | 761                           | 0.276     | 1/2,730    | 1.0                             |
| 1.25:1                                     | MnCl <sub>2</sub> | 396                           | 0.081     | 1/4,890    | 2.0                             |
| Novikoff hepatoma DNA polymerase- <i>β</i> |                   |                               |           |            |                                 |
| —  | MgCl <sub>2</sub> | 517                           | 0.103     | 1/5,020    | 1.0                             |
| 0.5:1                                      | MgCl <sub>2</sub> | 199                           | 0.014     | 1/14,100   | 2.8                             |
| 1.25:1                                     | MgCl <sub>2</sub> | 47                            | <0.001    | <1/47,000  | >9.4                            |
| Calf thymus DNA polymerase- <i>α</i>       |                   |                               |           |            |                                 |
| —  | MgCl <sub>2</sub> | 198                           | 0.044     | 1/4,500    | 1.0                             |
| 0.5:1                                      | MgCl <sub>2</sub> | 359                           | 0.023     | 1/15,800   | 3.5                             |
| 1.25:1                                     | MgCl <sub>2</sub> | 57                            | 0.001     | 1/57,000   | 12.7                            |

Assays were performed in a 50- $\mu$ l volume containing 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, divalent metal ion activator (2 mM MgCl<sub>2</sub> or 1 mM MnCl<sub>2</sub>), 1  $\mu$ g of poly(dA-T), 50  $\mu$ M dATP, 50  $\mu$ M [ $\alpha$ -<sup>32</sup>P]dTTP (8–20 cpm/pmol), 50  $\mu$ M [H]dGTP (11,920 cpm/pmol), 0.1–0.6 units of the indicated DNA polymerase, and *E. coli* SSB as shown. Incubation was at 37°C for 15 min (Pol I) or 60 min (*T<sub>4</sub>*, AMV,  $\alpha$ , and  $\beta$ ).

with DNA polymerases from AMV, Novikoff hepatoma ( $\beta$ ), and calf thymus ( $\alpha$ ) is not mediated by an increase in excision of incorrectly incorporated nucleotides, because these enzymes lack any proofreading exonuclease activity (2, 36, 37). Also, the improvement in accuracy is not correlated with enhanced polymerase activity. Thus, Pol I, *T<sub>4</sub>* DNA polymerase, and AMV DNA polymerase were stimulated by SSB, whereas DNA polymerase- $\beta$  was inhibited, yet the accuracy of each of these polymerases was increased. As with Pol III, the increase in accuracy of each enzyme was greatest at the highest concentration of SSB used.

**Effect of SSB on Fidelity with a Natural DNA Template.** Measurements of fidelity with synthetic homopolymers and heteropolymers are potentially subject to artifacts inherent in the repetitive sequences of these templates. We therefore examined the effect of SSB on the fidelity of DNA polymerases by using the recently developed  $\phi$ X174 fidelity assay (26, 27). In this assay,  $\phi$ X174 DNA containing an amber mutation in the gene *D/E* overlap is primed with a specific restriction endonuclease fragment and copied by a DNA polymerase *in vitro*. Certain incorrect minicorepairs at the amber site will produce reversion to wild type. The DNA is used to infect *E. coli* spheroplasts that are plated on indicator bacteria. The error rate is then determined from the reversion frequency for copied DNA when compared to an uncopied control. Biasing the substrate pool by a relative increase in an incorrect nucleotide—in this case, dATP—provides an error-prone condition that can be used for accurate quantitation of increased accuracy (26). The  $\phi$ X174 assay can be used to quantitate error rates for any DNA polymerase capable of utilizing single-stranded DNA as

a template. The inability of Pol III to copy long stretches of single-stranded DNA (38, 39) thus precludes measurements of fidelity with this enzyme in the  $\phi$ X174 assay.

The error rates of five different DNA polymerases in the presence and absence of *E. coli* SSB when a 5-fold excess of incorrect nucleotide was used are shown in Table 5. With each enzyme, SSB increased the accuracy of DNA synthesis severalfold. Similar results were obtained by using a balanced substrate pool (data not shown). As with poly(dA-T), the effect was dependent on the concentration of SSB (with Pol I). It should be noted, however, that the magnitude of the increase in fidelity with SSB did vary considerably, depending on the enzyme and SSB preparations used (data not shown). Because the accuracy of DNA polymerases without 3'  $\rightarrow$  5' exonucleases was increased to an extent similar to that of Pol I, it is likely that the effect of SSB is not mediated by enhanced proofreading.

#### DISCUSSION

The *in vitro* accuracy of DNA replication of  $10^{-7}$  to  $10^{-11}$  appears to be achieved by a multistep process (3). The free energy of discrimination between incorrect and correct base pairs accounts for an error rate of only  $10^{-3}$ . DNA polymerases enhance base selection to error rates of  $10^{-5}$  to  $10^{-8}$ . All the DNA polymerases used in these studies fall into this range. The experiments described here indicate yet another step in approaching *in vitro* accuracy. SSB enhances the accuracy of *in vitro* DNA synthesis in two independent assay systems by at least 10-fold. Thus, fidelity *in vitro* can approach  $10^{-7}$ . The magnitude of the increase correlates closely with *in vitro* studies (16–18) on the mutator and antimutator effects of mutations



Table 5. Effect of SSB on accuracy of DNA polymerase in copying natural DNA

| SSB/DNA   | Nucleotides per template | Reversion frequency ( $\times 10^{-4}$ ) | Error rate | Relative decrease in error rate |
|---|--------------------------|--|------------|---------------------------------|
| <i>E. coli</i> DNA Pol I                            |                          |  |            |                                 |
| —   | 0                        | 0.287                                    | —          | —                               |
| —   | 522                      | 3.12                                     | 1/459      | 1.0                             |
| 2.5:1   | 665                      | 1.32                                     | 1/1,260    | 2.7                             |
| 5:1   | 694                      | 0.918                                    | 1/2,070    | 4.5                             |
| 7.5:1   | 573                      | 0.693                                    | 1/3,200    | 7.0                             |
| <i>T<sub>4</sub></i> DNA polymerase                 |                          |  |            |                                 |
| —   | 0                        | 0.787                                    | —          | —                               |
| —   | 1154                     | 1.44                                     | 1/1,990    | 1.0                             |
| 5:1   | 838                      | 0.841                                    | 1/24,100   | 12.1                            |
| AMV DNA polymerase                                  |                          |  |            |                                 |
| —   | 0                        | 0.416                                    | —          | —                               |
| —   | 159                      | 22.2                                     | 1/80       | 1.0                             |
| 7.5:1   | 253                      | 1.40                                     | 1/1,320    | 22.0                            |
| NcoKoff hepatitis B DNA polymerase- $\beta$         |                          |  |            |                                 |
| —   | 0                        | 0.854                                    | —          | —                               |
| —   | 491                      | 1.16                                     | 1/1,590    | 1.0                             |
| 7.5:1   | 130                      | 0.640                                    | 1/4,390    | 2.8                             |
| Acute lymphocytic leukemia DNA polymerase- $\alpha$ |                          |  |            |                                 |
| —   | 0                        | 0.476                                    | —          | —                               |
| —   | 348                      | 1.42                                     | 1/1,390    | 1.0                             |
| 5:1   | 283                      | 0.766                                    | 1/4,480    | 3.2                             |

DNA polymerase reactions were performed in alkalized test tubes in a 50- $\mu$ l volume containing 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 1.5 mM  $MnCl_2$ , 500  $\mu$ M dATP, 100  $\mu$ M each of dGTP, dTTP, and  $[a-^{32}P]dTTP$  (400–1200 dpm/pmol), 0.2  $\mu$ g of  $\phi$ X174 and 300  $\mu$ g of  $\phi$ X174 DNA primed at a 5-to-1 molar ratio with *Hae* III restriction endonuclease fragment  $Z_{\alpha}$  (9.5–1.0 unit of the indicated DNA polymerase, and *E. coli* SSB. Reactions were incubated at 37°C for 5 min (Pol I and T<sub>4</sub>), 60 min ( $\alpha$  and  $\beta$ ), or 120 min (AMV) and stopped by addition of 1  $\mu$ l of 100 mM EDTA. Incorporation was determined from duplicate 2- $\mu$ l aliquots and the values expressed as total nucleotides per template were calculated as an average, assuming all molecules are initiated and copied to the same extent. The remaining reaction mixture was used to transfect *E. coli* spheroplasts, which were then plated as infective centers on permissive and nonpermissive indicator bacteria (28). The reversion frequency of  $\alpha$  and  $\beta$  to wild type was then used to calculate the error rate (27). In an effort to increase the sensitivity of the assay, error-prone conditions were used by blunting the substrate pools to contain a 5-fold excess of one incorrect nucleotide, dATP (26). Whereas the error rates for Pol I and AMV DNA polymerase are in agreement with published results (26, 40), the error rates with  $\alpha$ ,  $\beta$ , and T<sub>4</sub> polymerase quoted here are initial measurements in  $Mn^{2+}$ . The actual error rates in  $Mg^{2+}$ -activated reactions for the latter enzymes under optimal conditions are yet to be determined.

in *T<sub>4</sub>* gene 32 (SSB). The increased accuracy is independent of the use of error-prone conditions, because it is observed with either  $Mg^{2+}$  or  $Mn^{2+}$  and with biased or balanced nucleotide substrate pools.

At this time, no strong conclusions on the mechanism of enhanced accuracy can be formulated. The severalfold enhancement in fidelity observed with DNA polymerases lacking a proofreading exonuclease indicate that this mechanism cannot be the reason for increased accuracy with these enzymes. However, enhanced proofreading could have a role with enzymes exhibiting a 3'  $\rightarrow$  5' exonuclease. Many mechanisms could explain the increase in fidelity; the simplest of these is enhanced base-selection due to a template/SSB interaction, resulting in increased rigidity of the template. The potential importance of this interaction is emphasized by the observation that a Pol I/ $Mn^{2+}$  complex orients the glycosylic bond of the incoming substrate to a conformational angle of 90° (41)—that

found in double-helical DNA- $\beta$ . Such an enzyme-mediated conformational change would position the substrate for correct base-pairing with the template. However, in order to maximize such a conformational enhancement in base selection, the nucleotides in the template should be held rigidly. In this instance, it should be more likely that the incoming nucleotide, if incorrect, will be rejected by steric overlap of the hydrogen bonds between template and substrate (42). Thus, by increasing template rigidity, SSB could amplify base selection during DNA synthesis. Consistent with this hypothesis is the fact that the enhancement in accuracy for a given amount of SSB is not an absolute value but is proportional to the relative accuracy of the DNA polymerase used (Tables 4 and 5). Also, this hypothesis is supported by the 30–80% inhibition of noncomplementary nucleotide turnover observed with T<sub>4</sub> DNA polymerase in the presence of T<sub>4</sub> DNA binding protein (10).

In addition to DNA polymerase and SSB, genetic evidence indicates that other proteins contribute to high fidelity (18). Liu *et al.* demonstrated that  $\phi$ X174 DNA could be copied by a seven-protein T<sub>4</sub>-replicating complex with an accuracy approaching that achieved during  $\phi$ X174 replication in *E. coli* (9). The method of analysis presented here can potentially be extended to determine the contribution to fidelity of any putative replicative protein (43). For example, the true replicative form of Pol III, the holoenzyme (44), contains at least three subunits in addition to those present in Pol III ( $\alpha$ ,  $\epsilon$ , and  $\theta$ ), the form of the enzyme used here. Potentially, these additional subunits, in conjunction with binding protein as well as other proteins, may allow *in vitro* DNA synthesis to proceed with the observed *in vivo* accuracy.

A number of exogenous agents have been demonstrated to decrease fidelity of copying synthetic polynucleotide templates (refs. 3 and 45; unpublished results) and, most recently, natural DNA (26). Most of these agents have been designated as mutagens or carcinogens. However, until now no additions to the *in vitro* reaction have been found to increase fidelity. Thus, the large enhancement in accuracy of *in vitro* DNA synthesis with SSB is even more striking, and coupled with *in vivo* data (18–19) it strongly suggests that SSB contributes significantly to the accurate replication of genetic information.

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**Exhibit 2: De Vries et al., *Eur. J. Biochem.* 224:613-622 (1994)**

## The single-stranded-DNA-binding proteins (SSB) of *Proteus mirabilis* and *Serratia marcescens*

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The single-stranded-DNA-binding (SSB) proteins from *Proteus mirabilis* and *Serratia marcescens* were purified from overproducing *Escherichia coli* strains, which were devoid of their own *ssb* gene. The strains harboured an *endA* insertion mutation and a *xonA* mutation resulting in the absence of endonuclease I and exonuclease I activities from the preparations. The amino acid sequences of the SSB of all three species are nearly identical in the N-terminal parts of the proteins that contain the DNA-binding domain, but differ in the C-terminal parts. Both proteins have an apparent binding-site size of 65 and 35 nucleotides at high and low salt concentrations, respectively. The association-rate constant for binding to poly(dT) is  $3.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  for *P. mirabilis* SSB (*PmiSSB*) and  $3.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  for *S. marcescens* SSB (*SmaSSB*). These binding parameters are very similar to those of *E. coli* SSB (*EcoSSB*). The structural similarity of the proteins is also documented by the finding that they can exchange subunits among each other to form mixed tetramers.

The transcriptional regulation of the *ssb* and *uvrA* genes from *P. mirabilis* and *S. marcescens* in SOS-induced *E. coli* cells was studied using *lacZ* fusions. While the *uvrA* genes were inducible, there was no induction of the *ssb* genes transcribed divergently from the *uvrA* genes. Apparently, regions with nucleotide sequence similarity to the *E. coli* SOS-box preceding the *ssb* genes of *P. mirabilis* and *S. marcescens* had no gross effect on the transcription. Studies on growth of the cells and recovery from ultraviolet damage indicate that the heterologous SSB proteins support DNA replication and recombinational DNA repair of *E. coli* with the same efficiency as the *E. coli* SSB protein. Interactions with other *E. coli* proteins involved in these processes either do not occur, or are not impeded.

Single-stranded-DNA-binding (SSB) proteins are important components of the macromolecular DNA metabolism of all kinds of organisms from viruses to vertebrates. Most of our knowledge about these proteins comes from studies on two non-related representatives, the gene 32 protein of phage T4 (T4 gp32) and the *Escherichia coli* SSB protein (*EcoSSB*). *EcoSSB* is essential and has been shown to be involved in DNA replication, DNA repair and genetic recombination (for a review, see Meyer and Laine, 1990). By binding of SSB, single-stranded DNA is prevented from forming secondary structures and becomes resistant to nucleases.

Binding of SSB to single-stranded nucleic acids can easily be monitored by the intrinsic tryptophan fluorescence of the protein. For *EcoSSB* at 0.3 M NaCl it was found that 65 nucleotides of poly(dT) are required to saturate a single tetramer and lead to a fluorescence quench of 90% (Lohman and

Overman, 1985). Lowering the NaCl concentration leads to a change in binding modes, and at 0.05 M NaCl an apparent binding-site size of 33 nucleotides is observed with a fluorescence quench of 50% (Lohman and Overman, 1985). At 0.3 M NaCl the protein binds to the single-stranded poly(dT) in an almost-diffusion-controlled reaction (Urbanke and Schaper, 1990). For a review of biophysical properties of *EcoSSB* see Lohman et al. (1988), Greipel et al. (1989), and Lohman and Bujalowski (1990).

SSB proteins with a partial amino acid sequence identity to *EcoSSB* have been found to be encoded by a number of transmissible plasmids like the *E. coli* F-factor (Chase et al., 1983). Some of them were shown to complement *ssb* mutants of *E. coli* (Golub and Low, 1985, 1986; Howland et al., 1989; Porter and Black, 1991). The biological role of the plasmid SSB is still unknown.

We have recently reported the cloning and sequencing of two further bacterial *ssb* genes in addition to the *E. coli* gene, namely those of *Serratia marcescens* and *Proteus mirabilis* (de Vries and Wackernagel, 1993, 1994). In evolutionary history, these species have separated from *E. coli* about 200 million and 400 million years ago (Ochman and Wilson, 1987), and represent a relatively close and a most distant

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Abbreviations: SSB, single-stranded-DNA-binding protein; *EcoSSB*, SSB protein from *Escherichia coli*; *PmiSSB*, SSB protein from *Proteus mirabilis*; *SmaSSB*, SSB protein from *Serratia marcescens*; X-gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactosidase.

relative of *E. coli* among the enterobacteria. The SSB proteins of these species have 89% (*S. marcescens*) and 81% (*P. mirabilis*) amino acid sequence identity with the *E. coli* SSB.

Several authors have studied a possible regulation of the *ssb* gene of *E. coli* by the LexA repressor. Transcription from the *ssbP* promoter, which is adjacent to the SOS box of the *uvrA* gene (located close to *ssb* and transcribed in the opposite direction as *ssb*), was inducible, but was accompanied by a decreased transcription from the two non-inducible promoters, *ssbP<sub>uvr</sub>* and *ssbP<sub>uvr</sub>* (Brandana et al., 1985). Correspondingly, the relative rate of synthesis of SSB increased slowly, but there was no increase in the level of SSB measured in cell extracts (Perrino et al., 1987). In *P. mirabilis* and *S. marcescens*, the *ssb-uvrA* region is organized similarly to that in *E. coli*. In the *ssb* promoter regions of both organisms a region with nucleotide sequence similarity to the *E. coli* SOS consensus sequence was previously identified, which is not present in the corresponding region in *E. coli* (de Vries and Wackemagel, 1993; 1994).

In this study we investigated the regulation of transcription of the *ssb* and *uvrA* genes of *P. mirabilis* and *S. marcescens* cloned in *E. coli*, particularly a possible effect of the SOS-box-like sequences was examined. Some physiological properties of an *E. coli* *ssb*-deletion mutant containing the SSB proteins from *P. mirabilis* (*PmSSB*) or *S. marcescens* (*SmaSSB*) were also studied. Functions of the purified proteins with single-stranded nucleic acids were investigated using physicochemical methods. Further, the compatibility of SSB promoters from the different organisms was examined in a subunit-exchange test. In addition, the amino acid sequences of the enterobacterial proteins are compared to those of plasmid-encoded SSB. The data are discussed with regard to the biological roles of SSB proteins in DNA metabolism.

## MATERIALS AND METHODS

### Purification of SSB proteins

*PmSSB* and *SmaSSB* were purified from the newly constructed strain BT270 which carries a *sonA2* mutation (Phillips et al., 1988) and an *endA* null mutation (Table 1; Jekel and Wackemagel, 1994). In this strain the tetracycline-resistance determinant from pBR322 was inserted into the *Nsi*I site of the *endA* gene. The strain is therefore deficient for exonuclease I and endonuclease I and carries the chromosomal *ssb* deletion of RDP268 (Porter et al., 1990). The strain requires an *ssb*<sup>+</sup> helper plasmid for growth. The helper plasmid pACYC53b (Porter et al., 1990) was replaced by pSBH2e or pSBH4e (Table 1) which carry the *ssb* genes of *P. mirabilis* and *S. marcescens*, respectively. This was achieved by plasmid transformation and subsequent isolation of chloramphenicol-sensitive segregants, which had lost pACYC53b. The replacement was confirmed by restriction analysis of plasmid DNA isolated from the cells. *Eco*SSB was isolated from JM103 F<sup>+</sup> harbouring pSBH5e (Table 1).

The SSB proteins were purified by the method of Lohman et al. (1986a) with minor modifications from cells grown in a 10-l fermenter (Braun). The preparations were about 99% pure as determined by SDS/PAGE and staining with Silver Stain (Bio-Rad). The *PmSSB* and *SmaSSB* isolated from strain BT270 were free of nuclease activity on linear single-stranded and double-stranded M13mp19 DNA up to a concentration of 2 µg/µl (the highest concentration tested), as determined by agarose-gel electrophoresis of DNA, that was incubated with different amounts of the pro-

teins in 25 mM Tris, pH 7.5, 12 mM MgCl<sub>2</sub>, 3.5% glycerol for 2 h at 37°C. The proteins were stable in storage buffer [20 mM potassium phosphate, pH 7.5, 1 M NaCl, 1 mM EDTA, 60% (by vol.) glycerol] at -20°C for at least 6 months.

### Construction of *lacZ* fusions for determination of the promoter strength

Several restriction fragments of the *ssb-uvrA* intergenic regions from *E. coli*, *S. marcescens* and *P. mirabilis* (Fig. 1) were cloned in both orientations into the *Sma*I site of the promoter test plasmid pTL61T (Linn and St Pierre, 1990). Where necessary, single-stranded overhangs were removed by mung-bean nuclease digestion (Gibco BRL). In one orientation, the transcription towards *ssb* is measured (pTL-*ssb* plasmid series), in the other the transcription towards *uvrA* (pTL-*uvr* plasmid series). The fragments with the complete promoter-operator regions of the three species were also cloned into the single-copy-number vector λTL61 (Linn and St Pierre, 1990) using the *Eco*RI- and *Xba*I sites of the identical multiple-cloning sites in both vectors. In the phage vector, *Eco*RI and *Xba*I are unique sites. Since the single-stranded ends produced by these enzymes are not complementary, the inserts were ligated into the arms in the same orientation as they had in the plasmid vector (with respect to the *lacZ* gene). The correct location and orientation of DNA fragments in the clones was verified by restriction analysis.

For the purpose of measuring the SOS inducibility of the cloned promoters in the λ vector, the phage 21 cI repressor of this vector had to be replaced by a repressor which is not cleaved upon SOS induction by mitomycin C. This was achieved by crossing the λcI857 gene (Sussman and Jacob, 1962) coding for an SOS-induction-resistant, thermosensitive cI repressor into the promoter-containing phages. *E. coli* C600 was used as host for the phage crosses. The descendants of the cross were plated on 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal)-plates with soft-agar (0.7%) containing 120 µg X-gal/ml and *E. coli* BT124 (Table 1) as indicator. After incubation for 16 h at 37°C, clear blue plaques were isolated and purified twice as single plaques. The correct size and orientation of the inserts was verified by restriction of the phage DNA with *Eco*RI and *Xba*I. Corresponding to the plasmids, the promoter test phages were termed λTL-*ssb*20, λTL-*ssb*40 and λTL-*uvr*20, λTL-*uvr*40, λTL-*uvr*50 (Fig. 1).

### Lysogen formation

Formation of lysogens of BT124 and BT148 (Table 1) was carried out as described (Linn and St Pierre, 1990). Lysogens were identified on agar plates containing X-gal (40 µg/ml). To distinguish monolysogens from multiple lysogens, a terminase excision test (Mousset and Thomas, 1969) was carried out: exponentially growing cultures (2 ml; 29°C) of the lysogens at a titre of 2×10<sup>8</sup>/ml were brought to 10 mM MgSO<sub>4</sub> and superinfected with the heteroimmune λTL61 (phage 21 immunity) at an infection multiplicity of two. The cultures were further aerated for 2 h. Then two drops of chloroform were added and incubation was continued for 10 min. Descendant phages with λ immunity were titred on λTL61 lysogens of *E. coli* C600 (Table 1). Monolysogens produced about 5×10<sup>6</sup> phage, while multiple lysogens produced about 5×10<sup>7</sup> phage with λcI857 immunity/ml in this test. The β-galactosidase activity of identified monolysogens (about

Table 1. Strains, phage and plasmids used in this study.

| <i>E. coli</i> strains, phage and plasmids | Relevant genotype/description   | Source or reference             |
|--|---|---------------------------------|
| RDP268                                     | <i>Δ(szb-uvrA)</i> ; only viable with an <i>szb</i> <sup>+</sup> helper plasmid   | Porter et al. (1990)            |
| BT124                                      | <i>Δlac(U169)</i> ; as GW1000, but <i>recA</i> <sup>+</sup>   | Kenyon and Walker (1980)        |
| BT148                                      | <i>Δlac(U169)</i> ; as GW1000, but <i>recA</i> <sup>+</sup> , <i>lexAJ</i>  | Kenyon and Walker (1980)        |
| IM103 (F <sup>-</sup> )                    | <i>endA1</i> ; the strain was cured of the F factor by electroporation  | Yanish-Perron et al. (1985)     |
| BT270                                      | <i>szb</i> <sup>+</sup> : Km <sup>r</sup> , <i>tonA2</i> , <i>endA</i> <sup>+</sup> ; Tc <sup>r</sup> ; only viable with an <i>szb</i> <sup>+</sup> helper plasmid              | this work                       |
| <i>ΔTL61</i>                               | promoter cloning phage vector   | Linn and St Pierre (1990)       |
| <i>Δcl857</i>                              | <i>cl857</i> -repressor (thermosensitive)   | Sussman and Jacob (1962)        |
| <i>ΔTL61-cl857</i>                         | same as <i>ΔTL61</i> , but with the <i>Δcl857</i> -repressor  | this work                       |
| pTL61T                                     | pBR322-derived promoter cloning plasmid vector  | Linn and St Pierre (1990)       |
| pRE432                                     | mini-F-derived single-copy-number cosmid vector   | de Vries and Wackernagel (1992) |
| pBluescript II KS <sup>+</sup>             | high-copy-number vector   | Stratagene, Heidelberg, Germany |
| pACYC308                                   | <i>E. coli szb</i> <sup>+</sup> gene in pACYC184  | Porter et al. (1990)            |
| pSBH2e                                     | <i>P. mirabilis szb</i> <sup>+</sup> gene in pBluescript II KS <sup>+</sup>   | de Vries and Wackernagel (1994) |
| pSBH4e                                     | <i>S. marcescens szb</i> <sup>+</sup> gene in pBluescript II KS <sup>+</sup>  | de Vries and Wackernagel (1993) |
| pSBH5e                                     | <i>E. coli szb</i> <sup>+</sup> gene in pBluescript II KS <sup>+</sup>  | de Vries and Wackernagel (1993) |
| pSBL4                                      | <i>S. marcescens szb</i> <sup>+</sup> gene in pRE432  | de Vries and Wackernagel (1993) |
| pSBL5                                      | <i>E. coli szb</i> <sup>+</sup> gene in pRE432  | de Vries and Wackernagel (1993) |
| pTL-sb20                                   | <i>P. mirabilis</i> , bp 338–599 ( <i>RsaI</i> – <i>RsaI</i> )  | this work                       |
| pTL-sb21                                   | <i>P. mirabilis</i> , bp 480–623 ( <i>MseI</i> – <i>MseI</i> )  | this work                       |
| pTL-sb22                                   | <i>P. mirabilis</i> , bp 338–494 ( <i>RsaI</i> – <i>Sau3AI</i> )  | this work                       |
| pTL-uv20                                   | <i>P. mirabilis</i> , bp 599–338 ( <i>RsaI</i> – <i>RsaI</i> )  | this work                       |
| pTL-uv22                                   | <i>P. mirabilis</i> , bp 494–338 ( <i>Sau3AI</i> – <i>RsaI</i> )  | this work                       |
| pTL-sb40                                   | <i>S. marcescens</i> , bp 290–350 ( <i>Sau3AI</i> – <i>Sau3AI</i> )   | this work                       |
| pTL-sb42                                   | <i>S. marcescens</i> , bp 131–437 ( <i>HaeIII</i> – <i>RsaI</i> )   | this work                       |
| pTL-uv40                                   | <i>S. marcescens</i> , bp 550–290 ( <i>Sau3AI</i> – <i>Sau3AI</i> )   | this work                       |
| pTL-uv42                                   | <i>S. marcescens</i> , bp 437–131 ( <i>RsaI</i> – <i>HaeIII</i> )   | this work                       |
| pTL-sb50                                   | <i>E. coli</i> , bp 85 in <i>uvrA</i> sequence (Husain et al., 1986) to bp 276 in <i>szb</i> sequence (Sancar et al., 1981) ( <i>NarI</i> – <i>PvuII</i> , total length 524 bp) | this work                       |
| pTL-uv50                                   | <i>E. coli</i> , bp 276 in <i>szb</i> sequence (Sancar et al., 1981) to bp 85 in <i>uvrA</i> sequence (Husain et al., 1986) ( <i>PvuII</i> – <i>NarI</i> ; total length 524 bp) | this work                       |

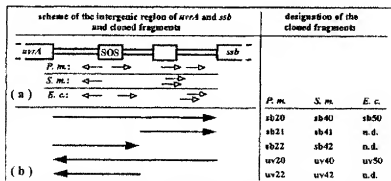


Fig. 1. Scheme of the intergenic region between *uvrA* and *szb* in *P. mirabilis*, *S. marcescens* and *E. coli*. (a) The start sites of the open reading frames of *uvrA* and *szb* are indicated. SOS represents the SOS boxes regulating the transcription of the *uvrA* genes. A second region with lower similarity to the *E. coli* SOS box consensus sequence is indicated by an empty box (in *E. coli*, only the left of these two regions is present). The location and direction of transcription of promoters in the three species is indicated by the open arrows. (b) The filled arrows indicate the fragments cloned into pTL61T and the direction of transcription measured (i.e. the orientation of the cloned fragment) in pTL61T. The designation of the cloned fragments is given for *P. mirabilis* (*P. m.*), *S. marcescens* (*S. m.*) or *E. coli* (*E. c.*); n.d., not determined.

370  $U/A_{260}$  of ) at a titre of  $10^9$ /ml) and multiple lysogens of BT124 for  $\lambda$ TL-sb40 (about 700  $U/A_{260}$  of 1) confirmed the reliability of the test. As expected, the lysogens were non-viable at 42°C due to induction of the prophage by inactivation of the cI857 repressor. The EcoRI-restriction pattern of the DNA of phage released by thermal induction was identical to that of the DNA of the phage used for lysogen formation.

#### Determination of ultraviolet sensitivity and generation time

The ultraviolet sensitivity and generation time of exponentially growing cultures of *E. coli* were determined as described (de Vries and Wackernagel, 1993, 1994).

#### Determination of $\beta$ -galactosidase activity and SOS induction

The  $\beta$ -galactosidase activity of all transcriptional fusions was determined by the method of Kenyon et al. (1982). The SOS response was induced by the addition of mitomycin C (Sigma) to the exponentially growing cultures (final concentration of 0.5  $\mu$ g/ml in *lexA*<sup>+</sup> strains; 0.1  $\mu$ g/ml in *lexA*<sup>-</sup> strains).

#### Binding of SSB proteins to single-stranded nucleic acids

Fluorescence measurements, stopped-flow experiments, and melting curves were carried out in a standard buffer containing 0.02 M potassium phosphate, pH 7.4, 0.1 mM EDTA and NaCl as indicated. In fluorescence experiments 100 ppm Tween20 were added.

Fluorescence measurements were carried out in a Schoeffel RRS1000 spectrofluorimeter at 22°C. Excitation was at 295 nm and emission was observed at 350 nm.

In inverse fluorescence titrations a mixture of protein and nucleic acid was added to the protein such that at all points the protein concentration was kept constant. Inverse titrations are often evaluated with respect to an apparent binding-site size using the intersection of the limiting slopes at low and high single-stranded DNA/protein ratios. In all titrations the absorbance of the solution was below 0.05 at the excitation wavelength to avoid inner filter effects. After each addition the solution was allowed to equilibrate for 60–600 s until no fluorescence change with time could be observed. Theoretical binding isotherms were calculated using the model of Schwarz and Watanabe (1983) for the binding of a multidentate ligand to a linear polymer as described earlier (Curth et al., 1993).

Melting curves were measured in a DMR10 (Zeiss) spectrophotometer in standard buffer and 0.1 M NaCl as described earlier (Augustinus et al., 1991). Temperature slopes were 20 K/h and no difference could be observed between heating and cooling curves.

Stopped-flow experiments were performed at 22°C in a modified version of a Durrum-Gibson stopped-flow apparatus and were evaluated using a model for irreversible binding of a multidentate ligand to a linear polymer, as described previously (Urbanke and Schaper, 1990).

Poly(dT) and poly(dA-dT) were purchased from Pharmacia. Poly(dT) had an average length of 1400 bases/strand as calculated from the sedimentation and diffusion coefficients measured by analytical ultracentrifugation and dynamic light scattering, respectively. Poly(dA-dT) showed a

broad distribution in length ranging from several hundred to 10000 bp as judged from agarose-gel electrophoresis. Concentrations of poly(dT) and poly(dA-dT) were determined photometrically with a molar absorption coefficient of 8600  $M^{-1}cm^{-1}$  at maximum (Urbanke and Schaper, 1990) and 6700  $M^{-1}cm^{-1}$  at 260 nm (Williams et al., 1983), respectively, and are given in monomer units throughout the text.

Protein concentrations are given in units of tetramers throughout the text and were determined using a molar absorption coefficient at 280 nm of 113000  $M^{-1}cm^{-1}$  as determined previously (Lohman and Overman, 1985) for EcoSSB. Since the predicted amino acid sequences indicate that EcoSSB, PmSSB and SmaSSB have no differences in aromatic amino acid composition no differences in the molar absorption coefficients at 280 nm are to be expected.

#### Amino acid sequencing

Amino acid sequencing was performed with the purified proteins with an Applied Biosystems 477A protein sequencer.

#### Subunit-exchange measurements

The proteins were diluted from stock solutions to a concentration of 100  $\mu$ M with SBP buffer [20 mM potassium phosphate, pH 7.5, 0.5 M NaCl, 1 mM EDTA, 20% (by vol.) glycerol]. Equal volumes of these solutions were mixed and incubated at room temperature for the indicated times. Samples were diluted with equal volumes of loading buffer (50% (by vol.) glycerol, 0.1% bromophenol blue). Samples containing 125 pmol of each protein were loaded onto an 0.5% agarose gel and electrophoresed in 20 mM Tris, pH 7.8, 0.4 mM NaAc, 0.2 mM EDTA as described earlier (Lohman et al., 1986b). The gel was stained with Coomassie Blue for 1 min and destained in 7% (by vol.) acetic acid at room temperature for several days.

## RESULTS AND DISCUSSION

#### Analysis of the expression and regulation of *ssb* and *uvrA* genes

The analysis of the nucleotide sequence of the intergenic region between *ssb* and *uvrA* had revealed two sequences in both *S. marcescens* and *P. mirabilis*, with similarity to the *E. coli* SOS box (de Vries and Wackernagel, 1993, 1994; EMBL accession numbers X65079, X65080). SOS boxes are operators where the LexA repressor binds regulating the expression of genes of the SOS regulon (Walker, 1984). In both species the nucleotide sequence similarity of the SOS-box-like sequence that overlaps with the *uvrA* promoter to the *E. coli* SOS consensus sequence (TACTGTATATA-ACAGTA) was high [TACTGTATATCCATTCAAGT (*S. marcescens*), TACTGTATATCCATTCAAGT (*P. mirabilis*)], and less high for the other one that overlaps with the *ssb* promoter region [GTGTGGTTTCCTACTCAGCG (*S. marcescens*), TCCGTTCTCAATGGAGAA (*P. mirabilis*)].

To determine the strength of the promoters and to identify a possible regulation by the LexA repressor, transcriptional *lacZ* fusions were constructed and expressed in *E. coli*. Fragments containing the entire intergenic region as well as smaller fragments containing either of the two SOS-box-like sequences were subcloned into the promoter-analytic vector pTL61T (Linn and St Pierre, 1990). Fig. 1 shows a scheme

Table 2.  $\beta$ -galactosidase activity of transcriptional fusions of *ssb* and *uvrA* promoters in pTL61T. The designation of the plasmids is described in Fig. 1. The determination of  $\beta$ -galactosidase activity was performed by the method of Kenyon et al. (1982). The induction factors were calculated by division of induced by uninduced  $\beta$ -galactosidase activity of the *lexA*<sup>+</sup> strain, separately for 1 h and 2 h after induction. The values in parentheses were determined similarly for the uninducible *lexA3* mutant. The data are means from three experiments ( $\pm$  the standard deviation).

| Plasmid  | $\beta$ -galactosidase activity of uninduced cultures after induction for |                |                | Induction factor in <i>lexA</i> <sup>+</sup> (in <i>lexA3</i> ) after induction for |                     |
|----------|---|----------------|----------------|---|---------------------|
|          | 0 h   | 1 h            | 2 h            | 1 h   | 2 h                 |
|          | 1000 U/A <sub>500</sub> of 1  |                |                |   |                     |
| pTL-sb20 | 9.0 $\pm$ 0.9   | 8.9 $\pm$ 0.8  | 12.0 $\pm$ 0.8 | 0.8 $\pm$ 0.1 (0.7)   | 0.6 $\pm$ 0.2 (0.7) |
| pTL-sb21 | 9.2 $\pm$ 2.2   | 9.9 $\pm$ 0.2  | 14.6 $\pm$ 0.5 | 0.9 $\pm$ 0.1 (0.8)   | 1.2 $\pm$ 0.1 (0.5) |
| pTL-sb22 | 6.0 $\pm$ 1.6   | 5.6 $\pm$ 0.7  | 9.4 $\pm$ 2.1  | 1.6 $\pm$ 0.1 (1.2)   | 1.4 $\pm$ 0.2 (0.7) |
| pTL-uv20 | 4.8 $\pm$ 0.1   | 4.2 $\pm$ 0.7  | 5.5 $\pm$ 1.1  | 1.3 $\pm$ 0.1 (1.0)   | 1.5 $\pm$ 0.1 (0.6) |
| pTL-uv22 | 5.0 $\pm$ 0.5   | 4.8 $\pm$ 0.2  | 8.1 $\pm$ 0.3  | 1.4 $\pm$ 0.3 (0.4)   | 1.1 $\pm$ 0.1 (0.7) |
| pTL-sb40 | 7.6 $\pm$ 0.8   | 9.3 $\pm$ 0.1  | 14.5 $\pm$ 1.0 | 1.0 $\pm$ 0.1 (0.8)   | 1.1 $\pm$ 0.1 (0.6) |
| pTL-sb41 | 9.0 $\pm$ 0.1   | 10.7 $\pm$ 0.4 | 15.3 $\pm$ 0.2 | 0.9 $\pm$ 0.1 (1.0)   | 1.0 $\pm$ 0.0 (0.6) |
| pTL-sb42 | 0.4 $\pm$ 0.0   | 0.6 $\pm$ 0.0  | 0.9 $\pm$ 0.0  | 1.1 $\pm$ 0.1 (0.7)   | 1.0 $\pm$ 0.0 (0.7) |
| pTL-uv40 | 6.6 $\pm$ 0.1   | 7.0 $\pm$ 0.6  | 9.6 $\pm$ 1.1  | 2.2 $\pm$ 0.4 (0.6)   | 2.6 $\pm$ 0.3 (0.6) |
| pTL-uv42 | 8.3 $\pm$ 1.5   | 6.8 $\pm$ 0.4  | 11.1 $\pm$ 0.7 | 2.0 $\pm$ 0.5 (0.6)   | 2.1 $\pm$ 0.0 (0.7) |
| pTL-sb50 | 7.1 $\pm$ 0.6   | 9.2 $\pm$ 0.4  | 15.4 $\pm$ 0.9 | 1.2 $\pm$ 0.2 (1.1)   | 0.9 $\pm$ 0.3 (0.9) |
| pTL-uv50 | 1.3 $\pm$ 0.2   | 1.3 $\pm$ 0.4  | 4.6 $\pm$ 0.4  | 3.2 $\pm$ 0.2 (1.2)   | 2.2 $\pm$ 0.3 (1.1) |
| pTL61T   | 0.7   | 0.7            | 1.2            | 1.0 (1.0)   | 0.9 (0.9)           |

of the various cloned regions and the designation of the plasmids containing the fragments. With each of these plasmids the  $\beta$ -galactosidase activity was determined under non-induced conditions and after SOS induction by mitomycin C (see Materials and Methods), both in a *lexA*<sup>+</sup> strain and a non-inducible *lexA3* mutant.

The  $\beta$ -galactosidase activity obtained with the pTL-sh plasmids from *P. mirabilis*, *S. marcescens* and *E. coli* in uninduced cells at a titre of about  $10^8$  ml was in the range of about 6–9000 U/A<sub>500</sub> of 1 with all of the pTL-sh plasmids (Table 2). Only pTL-sb42 gave a low level of activity as the promoterless vector pTL61T. It was concluded that no transcription towards *ssb* was initiated from the *S. marcescens* region containing the *uvrA* promoter and SOS operator sequences. In this respect, *S. marcescens* differs from *E. coli*, where transcription towards *ssb* is initiated by a promoter adjacent to the *uvrA* SOS box (Brandtsma et al., 1985).

Transcription towards *uvrA* produced 1300 U/A<sub>500</sub> of 1 from the *E. coli* promoter (pTL-uv50), 4800 from the *P. mirabilis* promoter (pTL-uv20) and 6600 from the *S. marcescens* promoter in pTL-uv40. Similar results were obtained when the smaller fragments with only the *uvrA* promoters were employed (Table 2). 2 h after induction with mitomycin C the cultures entered the stationary phase, and the  $\beta$ -galactosidase activity increased gradually with all of the plasmids.

The induction factors (level of induced activity divided by uninduced level) obtained with mitomycin C induction are shown in the right two columns of Table 2. In a *lexA*<sup>+</sup> background the *P. mirabilis* *ssb* promoter in pTL-sb22 was inducible (by a factor of 1.6). The *lacZ* expression from the larger fragment contained in pTL-sb20 was not inducible, rather it was slightly reduced after application of mitomycin C. A similar observation was made in *E. coli*, where the induction of the *ssbP*<sub>1</sub> promoter was accompanied by a decrease of the transcription from *ssbP*<sub>2</sub> and *ssbP*<sub>3</sub> (Brandtsma et al., 1985). The expression towards *ssb* in the other constructs with promoters of *E. coli*, *P. mirabilis* and *S. marcescens* was not inducible (Table 2).

In contrast, transcription towards *uvrA* was induced by mitomycin C in constructs with the *uvrA* promoters of *P. mirabilis* (by a factor of 1.5), *S. marcescens* (by a factor of 2.6) and *E. coli* (by a factor of 3.2), which is in accord with published data on *E. coli* (Kenyon and Walker, 1980). The inducibility was abolished in *lexA3* cells (in which the LexA repressor is not cleaved due to a mutation), indicating that the SOS boxes at the *uvrA* promoters of *P. mirabilis* and *S. marcescens* were recognized by the *E. coli* LexA repressor.

The observed absent or low SOS induction of the *ssb* genes may result from the use of the multicopy vector pTL61T, which might titrate the LexA repressor by the high number of SOS boxes/cell. Therefore, the insert fragments with the complete promoter regions were also cloned into the single-copy-number vector  $\lambda$ TL61. However, the results of  $\beta$ -galactosidase-activity determinations with the phage vector (Table 3) after SOS induction gave an induction pattern in *lexA*<sup>+</sup> and *lexA3* cells similar to that obtained with the multicopy vector. Again, the *ssb*-promoter regions of *S. marcescens* and *P. mirabilis* ( $\lambda$ TL-sb20 and  $\lambda$ TL-sb40) did not display any inducibility.

The results suggest that the SOS-box-like sequences preceding the *ssb* genes of *P. mirabilis* and *S. marcescens* have no gross effect on the *ssb* gene expression in SOS-induced *E. coli* cells. Possibly, the similarity of the SOS-box-like sequences of *P. mirabilis* and *S. marcescens* to the *E. coli* SOS consensus sequence is not sufficiently high to allow efficient binding of the *E. coli* LexA repressor. This would leave open the possibility that these putative SOS boxes have a regulatory function in *P. mirabilis* and *S. marcescens*. Expression of the *lacA* gene of *S. marcescens*, which is also preceded by an SOS-box-like sequence, is inducible in *S. marcescens*, but not in *E. coli* (Baill et al., 1990). The *uvrA* genes of *P. mirabilis* and *S. marcescens* are inducible in *E. coli*, although not to the same degree as the *E. coli* *uvrA* gene (Tables 2 and 3). The lower induction factors could result from deviations in the nucleotide sequence of the SOS boxes in *S. marcescens* and *P. mirabilis* from the *E. coli* SOS box consensus



**Table 3.  $\beta$ -galactosidase activity of transcriptional fusions of *ssb* and *uvrA* promoters in the single-copy-number vector  $\lambda$ TL61T.**  
For further details, see the legend to Table 2.

| Lysogen                      | $\beta$ -galactosidase activity of uninduced cultures after induction for |              |              |              | Induction factor in <i>lexA</i> <sup>+</sup> (in <i>lexA</i> 3) |                     |                     |
|------------------------------|---|--------------|--------------|--------------|---|---------------------|---------------------|
|                              | 0 h   | 1 h          | 2 h          | 3 h          | 1 h   | 2 h                 | 3 h                 |
|                              | U/A <sub>500</sub> of 1   |              |              |              |   |                     |                     |
| $\lambda$ TL- <i>ssb20</i>   | 400 $\pm$ 34  | 380 $\pm$ 36 | 470 $\pm$ 36 | 560 $\pm$ 88 | 0.9 $\pm$ 0.1 (0.9)   | 0.9 $\pm$ 0.0 (1.0) | 0.9 $\pm$ 0.1 (1.0) |
| $\lambda$ TL- <i>uvr20</i>   | 76 $\pm$ 04   | 86 $\pm$ 03  | 110 $\pm$ 07 | 140 $\pm$ 07 | 1.3 $\pm$ 0.1 (1.0)   | 1.4 $\pm$ 0.1 (1.0) | 1.5 $\pm$ 0.0 (1.0) |
| $\lambda$ TL- <i>ssb40</i>   | 370 $\pm$ 45  | 310 $\pm$ 32 | 380 $\pm$ 17 | 450 $\pm$ 40 | 1.1 $\pm$ 0.1 (1.1)   | 1.0 $\pm$ 0.1 (0.9) | 0.8 $\pm$ 0.2 (0.9) |
| $\lambda$ TL- <i>uvr40</i>   | 300 $\pm$ 09  | 280 $\pm$ 04 | 320 $\pm$ 04 | 330 $\pm$ 10 | 1.8 $\pm$ 0.0 (1.0)   | 3.1 $\pm$ 0.0 (1.0) | 3.6 $\pm$ 0.1 (1.0) |
| $\lambda$ TL- <i>uvr50</i>   | 140 $\pm$ 01  | 110 $\pm$ 10 | 130 $\pm$ 15 | 140 $\pm$ 12 | 1.7 $\pm$ 0.1 (0.8)   | 3.2 $\pm$ 0.1 (1.0) | 4.9 $\pm$ 0.1 (1.0) |
| $\lambda$ TL61- <i>c1857</i> | 71  | 58           | 78           | 84           | 1.1 (1.0)   | 1.0 (1.0)           | 1.0 (1.0)           |

sequence. The SOS regulons of the three enterobacterial species are apparently similar, but they are probably not identical. The determination of the nucleotide sequences of more SOS boxes from *S. marcescens* and *P. mirabilis* would be necessary for establishing the SOS consensus sequences for these species.

#### Comparison of the amino acid sequences of chromosomally and plasmid-encoded SSB proteins

In *E. coli* the N-terminal methionine residue of EcoSSB is cleaved post-translationally (Sancar et al., 1981). From the determination of the N-terminal amino acid sequences of *PmiSSB* and *SmaSSB* expressed in *E. coli* the same cleavage of methionine can be shown. The alignment of the amino acid sequences of the bacterial SSB proteins with those of five SSB proteins encoded by transmissible plasmids (Fig. 2) indicates that all eight proteins are nearly identical up to position 88. Amino acids that are presumed to be involved in tetramer formation (His55; Williams et al., 1984; Curth et al., 1991) or DNA binding (Trp40, Trp54, Phe60, Trp88; Merrill et al., 1984; Casas-Finet et al., 1987; Khamis et al., 1987; Curth et al., 1993) of the EcoSSB are found at identical positions within all eight proteins. The C-terminal third is more variable within each group, and between both groups the amino acid sequence similarity is low. From position 114–177, there are only three stretches of 4, 3 and 5 amino acids, starting at positions 136, 149, and 173, that are highly conserved in all eight SSB proteins. Other regions are highly conserved within a group, but are completely different between both groups. This pattern of regions with high and low amino acid sequence similarity may reflect the evolutionary relatedness of SSB, i.e. differences would indicate positions not essential for the structure and functioning of the proteins. However, it is possible that the regions with low or no sequence similarity between bacterial and plasmid-encoded SSB have different functions in both groups (Chase et al., 1983).

Compared to the other SSB proteins, the *E. coli* SSB contains an apparent insertion of six amino acids at positions 126–131 (the *S. marcescens* protein has a smaller insertion of three amino acids at the same position). This insertion could be a duplication of the nucleotide sequence that immediately follows, encoding the amino acid sequence GWGQPQ. Only three nucleotides are different, which results in the replacement of W by G. The duplication produces a further copy of the sequence GQFQ, which is one of the

three conserved regions in the C-terminus among all eight proteins.

#### DNA binding by the SSB proteins from *S. marcescens* and *P. mirabilis*

One of the most important biophysical properties of SSB proteins is their ability to destabilize double-stranded nucleic acids. Fig. 3 shows the destabilization of alternating poly(dA-dT) by both *SmaSSB* and *PmiSSB*. From the relative transition midpoints one sees directly that both proteins drastically destabilize poly(dA-dT) and that the double-stranded destabilizing ability of *SmaSSB* is somewhat higher than that of *PmiSSB*. Under the same conditions the transition midpoint of EcoSSB is at 48°C which is almost equal to that of *PmiSSB*.

For EcoSSB the binding to single-stranded nucleic acids has been investigated with various substrates with the affinity decreasing in the order poly(dT), phage single-stranded DNA (fd,  $\Phi$ X174), poly(dC), poly(dC,dT), poly(dA,dC), poly(rU), poly(dA), polyribonucleic acids (Overman et al., 1988; Greipel et al., 1989). Since poly(dT) shows the strongest binding to EcoSSB it is especially suited to measure the binding-site size. However, this strong binding prevents the determination of binding affinities. In inverse titrations at 0.3 M NaCl in standard buffer 65 nucleotides of poly(dT) are covered by a single EcoSSB tetramer leading to a fluorescence decrease of the protein by 90% (Lohman and Overman, 1985; Bujalowski and Lohman, 1986; Bujalowski et al., 1988). Fig. 4 shows fluorescence titrations for *SmaSSB* and *PmiSSB* under the same conditions. Binding-site size and fluorescence quench are similar to EcoSSB with only minor differences. For EcoSSB a binding-mode transition has been shown when changing the ionic environment from high salt ( $\geq 0.3$  M NaCl) to low salt ( $\leq 0.05$  M NaCl) where the apparent binding-site size decreases from 65 to 33 nucleotides/tetramer (Lohman and Overman, 1985; Bujalowski et al., 1988). A similar transition is observed for *PmiSSB* and *SmaSSB* (Fig. 5).

The kinetics of binding to poly(dT) can be observed by fluorescence changes in a stopped-flow experiment. Using a kinetic model for the binding of a multidentate ligand to a linear polymer described earlier (Urbank and Schaper, 1990) the association-rate constants for the binding of a protein tetramer to its binding site was determined to be  $(3.2 \pm 0.4) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  for *PmiSSB* and  $(3.4 \pm 0.4) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  for *SmaSSB*. These values do not differ significantly

|                | 1   | 10  | 20  | 30  | 40   | 50          | 60  | 70 |
|----------------|---|-----|-----|-----|------|-------------|-----|----|
| <i>E. coli</i> | ASRGVKEVILVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| <i>S. m.</i>   | ASRGVKEVILVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| <i>P. m.</i>   | ASRGVKEVILVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
|                | * * *   |     |     |     |      |             |     |    |
| <i>F</i>       | AVRGINKVILVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| pIP231a        | AVRGINKVILVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| pIP71a         | AVRGINKVILVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| R64            | SARGINKVILVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| ColB-P9        | SARGINKVILVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
|                | 80  | 90  | 100 | 110 | 120  | 130         | 140 |    |
| <i>E. coli</i> | LEKGSQVYIYVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| <i>S. m.</i>   | LEKGSQVYIYVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| <i>P. m.</i>   | LEKGSQVYIYVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
|                | ***   |     |     |     |      |             |     |    |
| <i>F</i>       | LEKGAQVYIYVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| pIP231a        | LEKGAQVYIYVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| pIP71a         | LEKGAQVYIYVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| R64            | LEKGAQVYIYVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
| ColB-P9        | LEKGAQVYIYVGRIGNDPPEVYIYFPGGAVALQVATSESWRKEKQTWHRVYLPKGLAFAVAGY |     |     |     |      |             |     |    |
|                | 150   | 160 | 170 | 177 | # aa | aa identity |     |    |
| <i>E. coli</i> | PQGGNQ...FSGGAQ...SRPQ...SAPAPSNKPFMDDDIFF                      |     |     |     | 177  | 100 %       |     |    |
| <i>S. m.</i>   | PQGGNQ...FSGGAQ...SRPQ...SAPAPSNKPFMDDDIFF                      |     |     |     | 175  | 89 %        |     |    |
| <i>P. m.</i>   | PQASQQ...FSGGAQ...SRPQ...SAPAPSNKPFMDDDIFF                      |     |     |     | 173  | 81 %        |     |    |
|                | ***   |     |     |     |      |             |     |    |
| <i>F</i>       | XPQAKGTXKGAATKGRGKAAQPEPQPPHGDYVGFDDIFF                         |     |     |     | 178  | 60 %        |     |    |
| pIP231a        | XPQAKGTXKGAATKGRGKAAQPEPQPPHGDYVGFDDIFF                         |     |     |     | 178  | 60 %        |     |    |
| pIP71a         | XSQKPK...KKGAKTGRGKAAQPEPQPPHGDYVGFDDIFF                        |     |     |     | 174  | 62 %        |     |    |
| R64            | ADAT...KKGAKTGRGKAAQPEPQPPHGDYVGFDDIFF                          |     |     |     | 174  | 59 %        |     |    |
| ColB-P9        | ADAT...KKGAKTGRGKAAQPEPQPPHGDYVGFDDIFF                          |     |     |     | 174  | 61 %        |     |    |

Fig. 2. Alignment of SSB proteins from enterobacteria and conjugative plasmids *F* (Chase et al., 1983), pIP231a, pIP71a, R64 (Ruvolo et al., 1991) and ColB-P9 (Howland et al., 1989). The alignment was performed with the program MULTALIN (Corpet, 1988) using the Dayhoff-matrix (Dayhoff, 1978) and a gap-penalty of 8. The sequences are arranged in two groups (bacterial and plasmid SSB). In each group, conserved amino acids are shown in bold type (\*). Positions of identity between both groups (amino acids which are identical among of at least 50% of the members of each group). The number of amino acids of each protein (# aa) and the percentage of amino acids identical to the *E. coli* protein (aa identity) are given.

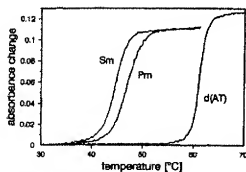


Fig. 3. Melting profiles of poly(dA-dT) and its complexes with *PmiSSB* and *SmaSSB*. 38.0  $\mu$ M poly(dA-dT) alone [d(AT)] and with 1.3  $\mu$ M *PmiSSB* (Pm) or *SmaSSB* (Sm) respectively in standard buffer at 0.1 M NaCl. Transition midpoints are at 61°C [d(AT)], 47°C (Pm), and 45°C (Sm). Absorbance changes were measured at 260 nm.

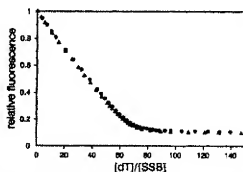


Fig. 4. Inverse fluorescence titration of *PmiSSB* (●) and *SmaSSB* (▲) with poly(dT). 0.31  $\mu$ M SSB protein (tetramer) were titrated with poly(dT) at 0.5 M NaCl in standard buffer. The binding-site size and fluorescence quench were calculated to be 66 nucleotides/tetramer and 89% for *PmiSSB*, and 65 nucleotides/tetramer and 90% for *SmaSSB*.

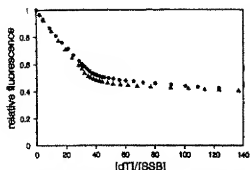


Fig. 5. Inverse fluorescence titration of 0.18  $\mu$ M *PmiSSB* (●) and 0.15  $\mu$ M *SmaSSB* (○) with poly(dT) at 1 mM NaCl, 1 mM potassium phosphate, pH 7.4, 0.1 mM EDTA and 100 ppm Tween20. The binding-site size was estimated from the limiting slopes to be 37 residues for *PmiSSB* and 36 residues for *SmaSSB*, respectively.

from the values determined for *EcoSSB* (Urbanke and Schaper, 1990).

#### Subunit exchange

Evidence for the structural and functional similarity of the various SSB proteins would be their ability to form mixed tetramers with each other. Different electrophoretic mobilities in a non-denaturing agarose gel can be used to separate homogeneous and mixed tetramers. SSB proteins, due to the highly charged C-terminus, have a net negative charge. *PmiSSB* having one acidic residue more than *EcoSSB* and *SmaSSB* can be separated from the latter two proteins. To obtain differences in electrophoretic mobility between *EcoSSB* and *SmaSSB* we used a deletion mutant of *EcoSSB* (*EcoSSB*Δ116–167) having the same net charge as wild-type *EcoSSB* but a reduced molecular mass and thus higher electrophoretic mobility. *EcoSSB*Δ116–167 cannot be separated, however, from *PmiSSB*.

As shown in Fig. 6 the different SSB proteins form mixed tetramers when incubated for 65 h at room temperature. In a time-resolved exchange experiment between *PmiSSB* and *SmaSSB* the first mixed tetramers appeared after 12 h (data not shown). There is no preference for the homotetramers in the exchange experiments. We therefore conclude that the mechanism of interaction in forming the tetramer must be virtually identical in all SSB proteins investigated. If the exchange was statistically random, a 1:1 mixture of SSB proteins A and B should form five types of tetramers (A<sub>4</sub>, A<sub>3</sub>B, A<sub>2</sub>B<sub>2</sub>, AB<sub>3</sub>, and B<sub>4</sub>) in a binomial distribution 1:4:6:4:1. This behaviour has been shown for lactate dehydrogenase (Markert, 1963), which is a tetramer formed by two electrophoretically distinguishable subunits. The relative intensities of the different bands in Fig. 6 show, however, that the amount of the A<sub>2</sub>B<sub>2</sub> type is far more than 50% larger than that of the corresponding A<sub>3</sub>B or AB<sub>3</sub> heterotetramers. This observation can be explained if the exchange is preferentially between dimers and not monomers. This explanation agrees well with the fact that *EcoSSB* crystals show a D<sub>2</sub> symmetry (Hilgenfeld et al., 1984) and suggest a dimer-of-dimers structure for the tetramer. Tetramers which contain two dimers of two different species could then very well be more prominent than 1:3 mixed tetramers. One might speculate that different structural domains in the monomer might be responsible for

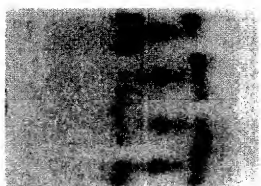


Fig. 6. Subunit exchanges between different SSB tetramers. Agarose-gel electrophoresis of different proteins mixed together in equimolar amounts and incubated at 23°C for 65 h, or put immediately onto the gel (0 h), and unincubated proteins. From top to bottom: *PmiSSB* with *EcoSSB*, 0 h; *PmiSSB* with *EcoSSB*, 65 h; *EcoSSB* alone; *PmiSSB* alone; *PmiSSB* with *SmaSSB*, 65 h; *PmiSSB* with *SmaSSB*, 0 h; *SmaSSB* alone; *SmaSSB* with *EcoSSB*Δ116–167, 65 h; *SmaSSB* with *EcoSSB*Δ116–167, 0 h; *EcoSSB*Δ116–167 alone; *EcoSSB* with *EcoSSB*Δ116–167, 65 h; *EcoSSB* with *EcoSSB*Δ116–167, 0 h.

dimer and tetramer formation. Experimental results which show no detectable amounts of dimers in the dissociation of *EcoSSB*H55Y (*ssb-1*) (Bujalowski and Lohman, 1991) do not contradict this interpretation since in both cases the dimeric state is only a transition state in either dissociation or subunit exchange and is not significantly populated compared to monomers or tetramers. Furthermore the compatibility of the SSB subunits from different species differing mainly in their C-terminal sequence and that of *EcoSSB*Δ116–167 indicates that tetramer formation is independent of the C-terminal amino acids.

#### Complementation of *E. coli* mutants

by the *ssb* genes of *P. mirabilis* and *S. marcescens*

The construction of an *ssb* deletion mutant, which is viable only when bearing a helper plasmid with an *ssb* gene (Porter et al., 1990) has made complementation studies with heterologous *ssb* genes possible (no *EcoSSB* is present). Previously, the presence of *ssb-1* or *ssb-113* alleles (point mutations) in *E. coli* precluded correct interspecies complementation studies. The deletion mutant was used to determine the generation time and ultraviolet resistance of *E. coli* cells with *PmiSSB* or *SmaSSB*, as measures for the function of these proteins in DNA replication and repair.

The generation time of *E. coli* RDP268 ( $\Delta$ *ssb*) carrying the *S. marcescens ssb* gene on the single-copy-number plasmid pSBL4 (Table 1) was 20.7  $\pm$  0.8 min, compared to 20.9  $\pm$  0.6 min with the *E. coli ssb* gene on the same vector (plasmid pSBL5; Table 1). With *PmiSSB* the generation time was 20.5  $\pm$  0.5 min (de Vries and Wackernagel, 1994). Thus, both heterologous SSB proteins can efficiently replace the *EcoSSB* in its function in DNA replication.

The ultraviolet sensitivity of *E. coli* RDP268 with pSBL4 or pSBL5 is shown in Fig. 7. In the *ssb-uvrA* deletion mutant, the recovery from ultraviolet damage depends on recombinational repair due to the lack of the UvrA protein required for excision repair. *SmaSSB* promotes DNA repair in *uvrA* cells

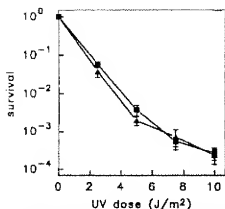


Fig. 7. Ultraviolet sensitivity of *E. coli* *uvrA-ssb* (RDF268; Table 1) with the *ssb* genes of *S. marcescens* (located on pSBL4; Δ) or *E. coli* (located on pSBL5; ■). The curves are means from three experiments. The error bars indicate standard deviations.

with the same efficiency as *EcoSSB*. This was also the case for *PmiSSB* (de Vries and Wackernagel, 1994). Thus, the differences in the amino acid sequences of the three enterobacterial SSB have no measurable effect on replication and recombinational repair in *E. coli*. The high similarity of the physicochemical properties of the proteins (see above) is probably the basis for the full support of replication and recombinational DNA repair in *E. coli* by the three proteins.

If interactions of SSB with other components of the macromolecular DNA metabolism exist, they seem not to be species specific, because the function of the *EcoSSB* can be fully replaced by *SmaSSB* or *PmiSSB*. In previous experiments, in which other enzymes of the macromolecular DNA metabolism were exchanged between the three species, evidence for species-specific interactions between *RecA* and *RecBCD* enzymes was obtained (Rinken et al., 1991; de Vries and Wackernagel, 1992). The tolerance for non-cognate SSB proteins as observed here would also be expected if no interactions of SSB with other enzymes exist.

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